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<p>(54) Title: ANTIBODIES TO PORCINE CD34 POSITIVE CELLS AND USES THEREFOR (57) Abstract Porcine CD34, oligonucleotides encoding the porcine CD34, porcine CD34 antibodies and methods of making and using each are disclosed.</p> <p style="text-align: right;">BEST AVAILABLE COPY</p>		

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ANTIBODIES TO PORCINE CD34 POSITIVE CELLS
AND USES THEREFOR

This invention relates to the field of antibodies that are specific to cell surface antigens and more particularly to such antibodies and compositions and methods that are useful for recovering porcine hematopoietic progenitor cells; in particular, stem cells. The invention further relates to isolated porcine cells which are recognized by an antibody of the invention and to the use of such porcine cells.

Organ procurement currently poses one of the major problems in organ transplantation, as the number of patients requiring transplants far exceeds the number of organs available. Despite some promising results with non-human primate-to-human xenotransplantation studies, it is very unlikely that, in the future, there will be widespread use of non-human primate organs. Firstly, chimpanzees and baboons are endangered species and there is much societal opposition to the use of such animals for experimental purposes. Secondly, there is concern over the transmission of non-human primate diseases when organs from such species are used in human transplants. It has been recognized that the use of swine organs for xenogeneic transplantation is an attractive

alternative source for organs. There is a close similarity between swine and humans with regards to parameters relevant to transplantation, including renal, cardiovascular and pulmonary physiology (Tumbleson, *Swine in Biomedical Research*, New York Plenum Press, 1986). Pigs are frequently sacrificed for human consumption; most swine diseases are unlikely to cause serious problems to humans and pigs are relatively easy to breed. Specifically, the organs of miniature swine seem appropriate for use as xenografts. While the weight of domestic swine are frequently in excess of 1,000 lb., the weight of the miniature swine is compatible with the weight of many adults, approximately 200 lb.

The second major problem regarding current transplantation procedures is the need for chronic immunosuppression in order to maintain the transplanted organ. The prolonged use of immunosuppressive agents, such as cyclosporin A, leads to renal dysfunction and may lead to graft failure. Much effort continues to be expended on means to induce immunologic tolerance which would obviate the necessity for chronic use of immunosuppression agents.

Tolerance to self major histocompatibility (MHC) antigens occurs during T cell maturation in the thymus (McDuffie et al., *J. Immunol.*, 141:1840, 1988). Exposure of the immune system to MHC antigens during ontogeny can cause the immune system to become tolerant to those antigens (Billingham, et al., *Nature* 172:603, 1953). Tolerance across major histocompatibility antigens has been achieved through the development of mixed bone marrow chimeras. It is desirable that the bone marrow population to be administered be enriched for the hematopoietic stem cells, since it is considered that it is this source of cells which will lead to prolonged bone marrow chimerism.

The development of monoclonal antibodies (mABs) to antigenic determinants on human hematopoietic cells has allowed identification and purification of subsets of marrow that contain precursor cells. Antibodies to CD34 antigen have been of interest because they react with a minor subset of cells that contains virtually all of the precursors of colony forming cells that can be detected in long-term marrow cultures. The CD34+ population of progenitor cells have been presumed to contain candidate stem cells. Indeed, populations enriched for CD34+ cells can engraft lethally irradiated baboons (Berenson et al., *J. Clin. Invest.*, 81:951, 1988).

Much has been learned in the past few years regarding the molecular biology of CD34 (Sutherland and Keating, *J. Hematotherapy*, 1:115-129, 1992). Both the human and murine cDNA genes have been cloned and sequenced. Genomic clones have been isolated and have demonstrated that both the human and murine genes are approximately 28 kb long. The human CD34 cDNA sequence predicts a molecule of 40 kDA with a maximum of nine potential N-linked glycosylation sites. The native molecule is predicted to contain a considerable number of O-linked glycans. In fact, over 35% of the 145 amino acids in the amino-terminal domain are either serine or threonine residues. The cluster of O-linked glycans in this domain may induce an extended structure to the molecule. Between the amino-terminal domain of potential heavy glycosylation and the transmembrane domain is a cysteine-rich region of 66 amino acids, which probably exhibits a globular conformation. The cytoplasmic domain of 73 amino acids contains several potential phosphorylation sites for a variety of protein kinases. The murine CD34 cDNA gene was isolated, using a probe corresponding to the human sequence, by using low stringency hybridization conditions. Both the murine and human cDNA sequences have significant homologies.

The intracellular domains are >90% identical, the transmembrane and proximal extracellular domains and the cysteine - rich region are also very similar (>75% and >70% identical, respectively). The amino-terminal domains of about 145 amino acids are only 45% sequence identical.

In accordance with one aspect of the present invention there is provided an antibody against porcine CD34 antigen.

In accordance with another aspect there is provided an antibody which can be used for recovery of CD34⁺ porcine cells.

In accordance with another aspect of the invention, there is provided a process for recovering CD34⁺ porcine cells, preferably from porcine bone marrow.

In accordance with another aspect of the present invention, there is provided a population of CD34⁺ porcine cells, in particular recovered from swine bone marrow or swine cord blood, preferably swine bone marrow.

In accordance with still a further aspect of the present invention, there is provided at least one polypeptide which can be used to produce an antibody which recognizes CD34⁺ positive porcine cells and a polynucleotide encoding such polypeptide.

In accordance with yet a further aspect of the invention, porcine CD34⁺ positive cells are employed to treat a host in particular a human patient who is to receive a porcine graft.

The porcine CD34⁺ cells are particularly employed to produce bone marrow mixed chimerism in a human patient to aid in acceptance of a porcine graft.

Antibodies against porcine CD34⁺ cells may be produced by use of a polypeptide of the invention or a peptide fragment thereof, which fragment includes at least one porcine CD34⁺ epitope.

Thus, for example, the antigen may be a soluble polypeptide which is at least 95% identical to the soluble portion of the porcine CD34⁺ antigen or may be a fragment of such polypeptide which fragment alone or in admixture with an appropriate protein is capable of generating antibodies against CD34⁺ cells.

In this respect, in accordance with an aspect of the present invention there is provided a polypeptide which is at least 95% identical to the mature polypeptide (polypeptide without the signal or leader sequence and including the transmembrane portion) and/or to the polypeptide with the signal or leader sequence which is encoded by the DNA included in ATCC Deposit No. 97,143, 97,144 or 97,145, filed on May 12, 1995.

The cDNA of clone 1Ax2-3 (ATCC 97,143) and the deduced polypeptide sequence encoded by such DNA of clone 1Ax2-3 is shown in Figure 1. The deduced polypeptide sequence encoded by the DNA in each of clones 1Bx2-4 and 1Cx2-5 is provided by reference to Table 1 and Figure 1.

Table 1

Changes from sequence of 1Ax2-3

<u>1Bx2-4</u> <u>NA POS</u>	<u>Substitute</u>	<u>Amino Acid Change</u>
202	A for G	ALA --> THR

405	G for T	No Change
596	G for A	HIS --> ARG
709	G for A	LYS --> GLU
812	C for T	LEU --> PRO

1Cx2-5NA POS

	<u>Substitute</u>	<u>Amino Acid Change</u>
202	A for G	ALA --> THR
405	G for T	No Change
597	C for T	No Change
831	G for A	THR --> ALA

In accordance with a further aspect of the present invention, there is provided a polypeptide which is at least 95% identical to the soluble portion of the polypeptide encoded by the DNA in the deposited clones (the soluble portion is the encoded polypeptide without the leader or signal portion and without the transmembrane portion).

The present invention further relates to a peptide fragment of such above polypeptides which peptide fragment includes an epitope which produces an antibody which recognizes porcine CD34⁺ cells.

The present invention further relates to polynucleotides which encode polypeptides which are at least 95% identical to the polypeptide having the deduced amino acid sequence of Figure 1 which includes amino acids 1 to 313, or amino acids -31 to 313 or amino acids 1 to 275, or amino acids -31 to 275, or to fragments of such polypeptides which fragments include at least one epitope of porcine CD34⁺ antigen.

As hereinabove indicated the polypeptides and peptide fragments of the present invention are employed to produce antibodies which recognize CD34⁺ porcine cells. Such

antibodies may be produced by use of a recombinant cell which expresses a polypeptide of the present invention which includes the transmembrane portion, whereby the recombinant cell includes such polypeptide on the surface thereof. Such recombinant cell may be employed for producing an antibody which recognizes CD34⁺ cells by procedures known in the art; for example as described in Example 3.

Alternatively, the soluble portion of the polypeptides of the present invention may be produced by recombinant techniques and such soluble polypeptide can be used for producing an antibody which recognizes porcine CD34⁺ cells by procedures known in the art; e.g., as described in Example 2.

In accordance with a further embodiment, a peptide fragment of the polypeptides of the present invention, for example, one which is chemically synthesized may be combined with a suitable protein, with such combination then being employed as an immunogen for producing antibodies which recognize porcine CD34⁺ cells. Thus, for example, the following peptides which are from regions of the polypeptide encoded by the DNA contained in clone 1Ax2-3 which regions form disulfide bonded loops may be used in preparing an immunogen for producing an antibody which recognizes porcine CD34⁺ cells:

Peptide 1: EVKCAQIKEVKLIQGIC

Peptide 2: ISGCEKFKKDNGEKLQMILC

The antibodies of the present invention may be employed for recovering porcine CD34⁺ cells from porcine bone marrow by procedures similar to those employed for recovering CD34⁺ cells from human bone marrow. Thus, for example, the antibodies may be supported on a suitable support by procedures known in the art for such purpose. The antibodies

may be physically supported, for example, by adsorption or through an appropriate chemical linkage. The antibodies may be supported, for example, by direct binding to a resin, such as Affigel 10 or 15, and the resulting affinity matrix used for purification of CD34⁺ cells. Thus, the antibodies of the present invention may be used in a column (an affinity column) to separate porcine CD34⁺ cells from porcine bone marrow.

The swine CD34⁺ cells may be used to produce mixed chimerism for inducing tolerance to an organ or tissue which is to be transplanted from swine into another species; in particular, a human. The published PCT application corresponding to PCT Application U.S. 93/00184 describes such a procedure using swine bone marrow and the porcine CD34⁺ cells of the present invention may be used in place of the swine bone marrow.

Published PCT Application corresponding to U.S. 94/12522 describes recovery of stem cells from swine cord blood, and the antibodies of the present invention are suitable for use in such a procedure.

Th antibodies may also be employed in an assay for determining the presence of porcine CD34⁺ cells using known assay procedures. For example, the antibody may be employed to determine whether or not mixed chimerism has occurred by assaying for the presence of porcine CD34⁺ cells in a host who has received such cells.

Thus, for example, such an assay may be effected by one of a variety of assay techniques generally referred to as immunoassays to detect the presence of porcine CD34⁺ cells.

Thus, in accordance with the present invention, there is provided one or more antibodies which are antibodies against one or more of the polypeptides encoded by DNA of one or more of the deposited clones. Such antibodies may be used to isolate porcine cells and in particular porcine cells from porcine bone marrow. The present invention further relates to porcine cells which are recognized by one or more of such antibodies. Such porcine cells are enriched for porcine hematopoietic progenitor cells and in particular stem cells.

The isolated porcine cells which are recognized by the antibodies of the present invention are referred to as porcine CD34⁺ cells in that the polypeptides of the present invention are believed to have at least a 95% identity to the porcine CD34⁺ antigen.

Accordingly, the present invention provides an antibody (or fragment or derivative thereof) and preferably, an antibody (or fragment or derivative thereof) which binds to porcine CD34⁺ cells, particularly to porcine CD34⁺ hematopoietic progenitor cells, e.g. stem cells.

The antibodies of the present invention have the characteristics of binding to an epitope of porcine CD34 antigen (e.g. CD34 positive porcine hematopoietic progenitor cells).

In accordance with another aspect of the present invention there is provided a method of preventing and/or inhibiting an immune response in human patients to a swine graft by treating the human recipient with CD34⁺ porcine cells.

Another aspect of the invention provides a method of inhibiting an immunogenic response in a xenogeneic

transplantation host, such as a human recipient, to a porcine organ or porcine tissue by introducing a porcine CD34⁺ enriched cell population to the intended recipient prior to introduction of the porcine transplant organ or tissue.

The antibodies of the invention can be, for example, polyclonal, monoclonal, chimeric, humanized or single chain antibodies, or Fab fragments. Various procedures known in the art may be used for the production of polyclonal antibodies. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler and Milstein, 1975, Nature 256:495-497). Techniques described for the production of single-chain antibodies (U.S. Patent 4,946,778) can be adapted to produce porcine CD34-specific single-chain antibodies.

The antibody against CD34⁺ porcine cells is preferably a murine antibody; however, the antibody may be produced in other species; e.g., rat or bovine.

One aspect of the present invention provides isolated nucleic acid molecules encoding polypeptides which produce antibodies against porcine CD34⁺ cells (including mRNAs, DNAs, cDNAs, genomic DNAs) as well as analogs and fragments thereof which produce polypeptides capable of generating such antibodies.

Another aspect of the invention provides porcine CD34⁺ cells that preferentially enhance the development of mixed bone marrow chimerism between porcine bone marrow cells and bone marrow cells of other species in particular a primate, and to DNA sequences encoding polypeptides which can be used

to produce antibodies which will specifically recognize the porcine CD34 antigen.

Figure 1 shows the nucleic acid sequence and deduced amino acid sequence of a polynucleotide and polypeptide of the present invention. The mature polypeptide (without signal sequence) begins at amino acid 1. The transmembrane portion of the polypeptide is putatively identified as beginning at amino acid 276.

The term "isolated" means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or DNA present in a living animal is not isolated, but the same polynucleotide or DNA, separated from some or all of the coexisting materials in the natural system, is isolated. Such DNA could be part of a vector and/or such polynucleotide could be part of a composition, and still be isolated in that such vector or polynucleotide is not part of its natural environment.

It is also advantageous that the sequences be in "purified" form. The term "purified" does not require absolute purity; rather, it is intended as a relative definition. The cDNA clones are obtained via manipulation of a partially purified naturally occurring substance (messenger RNA). By conversion of mRNA into a cDNA library, pure individual cDNA clones can be isolated from the synthetic library by clonal selection. Thus, creating a cDNA library from RNA and subsequently isolating individual clones from that library results in an approximately 10^6 fold purification of the native message. Purification of starting material or natural material to at least one order of magnitude, preferably two or three orders, and more preferably four or five orders of magnitude is expressly contemplated.

Furthermore, claimed polynucleotide which has a purity of preferably 0.001%, or at least 0.01% or 0.1%; and even desirably 1% by weight or greater is expressly contemplated.

The polynucleotide of the present invention may be in the form of RNA or in the form of DNA, which DNA includes cDNA, genomic DNA, and synthetic DNA. The DNA may be double-stranded or single-stranded, and if single stranded may be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes the polypeptides may be identical to the coding sequences specifically described or that of the deposited clone or may be a different coding sequence which coding sequence, as a result of the redundancy or degeneracy of the genetic code, encodes the same polypeptides as the described DNA or the deposited cDNA.

The polynucleotides which encode for the polypeptide of the invention include, but are not limited to: only the coding sequence for the mature polypeptide; the coding sequence for the mature polypeptide and additional coding sequence such as a leader or secretory sequence; the coding sequence for the mature polypeptide (and optionally additional coding sequence) and non-coding sequence, such as introns or non-coding sequence 5' and/or 3' of the coding sequence for the mature polypeptide; the coding portion for the mature polypeptide without the transmembrane portion, etc.

The present invention further relates to variants of the hereinabove described polynucleotides which encode for fragments, analogs and derivatives of the polypeptides having the deduced amino acid sequence of the mature polypeptides of the invention. The variant of the polynucleotide may be a naturally occurring allelic variant of the polynucleotides or a non-naturally occurring variant of the polynucleotides.

Thus, the present invention includes polynucleotides encoding the same polypeptides specifically described as well as variants of such polynucleotides which variants encode for a fragment, derivative or analog of such polypeptides. Such nucleotide variants include deletion variants, substitution variants and addition or insertion variants.

As hereinabove indicated, the polynucleotides may have a coding sequence which is a naturally occurring allelic variant of the coding sequences specifically described. As known in the art, an allelic variant is an alternate form of a polynucleotide sequence which may have a substitution, deletion or addition of one or more nucleotides, which does not substantially alter the function of the encoded polypeptide.

The deposit(s) referred to herein [ATCC designations: ATCC 97143 (DNA plasmid 1AX2-3); ATCC 97144 (DNA plasmid 1BX2-4); and ATCC 97145 (DNA plasmid 1CX2-5)] will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited materials, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to polypeptides which have the deduced amino acid sequence of Figure 1 or which have the amino acid sequence encoded by the deposited

cDNA, as well as fragments, analogs and derivatives of such polypeptide.

The terms "derivative" and "analog" when referring to the polypeptides encoded by the deposited cDNA, means a polypeptide which retains essentially the same biological function or activity as such polypeptide, i.e., is capable of producing an antibody against porcine CD34⁺ cells.

The polypeptides of the present invention may be recombinant polypeptides, natural polypeptides or synthetic polypeptides, preferably recombinant polypeptides.

The fragments, derivatives or analogs of the polypeptides encoded by the deposited cDNAs may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence, or (v) one in which a T helper cell epitope is fused to a CD34⁺ fragment in order to increase immunogenicity. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The polynucleotides of the present invention may be employed for producing polypeptides by recombinant techniques. Thus, for example, the polynucleotide may be included in any one of a variety of expression vectors for expressing a polypeptide. Such vectors include chromosomal, nonchromosomal and synthetic DNA sequences, e.g., bacterial plasmids; baculovirus; yeast plasmids; etc. However, any other vector may be used as long as it is replicable and viable in the host.

The appropriate DNA sequence may be inserted into the vector by a variety of procedures. In general, the DNA sequence is inserted into an appropriate restriction endonuclease site(s) by procedures known in the art. Such procedures and others are deemed to be within the scope of those skilled in the art.

The DNA sequence in the expression vector is operatively linked to an appropriate expression control sequence(s) (promoter) to direct mRNA synthesis. As representative examples of such promoters, there may be mentioned: SV40 promoter, the CMV promoter and other promoters known to control expression of genes in prokaryotic or eukaryotic cells or their viruses. The expression vector also contains a ribosome binding site for translation initiation and a transcription terminator. The vector may also include appropriate sequences for amplifying expression.

In addition, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells such as

dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, or such as tetracycline or ampicillin resistance in E. coli.

The vector containing the appropriate DNA sequence as hereinabove described, as well as an appropriate promoter or control sequence, may be employed to transform an appropriate host to permit the host to express the protein.

As representative examples of appropriate hosts, there may be mentioned: bacterial cells, such as E. coli, yeast cells, animal cells such as CHO, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein.

The following Examples illustrate the invention in various of its aspects without being a limitation on its scope.

Example 1

Isolation and Sequencing of Porcine CD34 cDNA

Materials and Methods:

Enrichment of porcine bone marrow cells

Porcine bone marrow cells obtained from donor animals from a herd of partially inbred mini-swine were harvested from the femurs and humeri by aseptically scraping with bone currettes and flushing the cavity with Dulbecco's modified saline without calcium and magnesium containing 5% citrate-phosphate-dextrose solution (Sigma, St. Louis, MO) and gentamicin (20 ug/ml, Gibco, Grand Island, NY). Cells were layered over Histopaque (sp. gr. 1.077, Sigma) and centrifuged at 400 X g for 25 minutes. Low density cells were collected, washed, resuspended in Iscove's Modified Dulbecco's Media (IMDM, Gibco), 10% fetal bovine serum (FBS),

Dnase (50 u/ml, Sigma), placed in tissue culture flasks (Costar, T150) at 5×10^6 /ml and allowed to adhere for 60 minutes at 37 C, 5% CO₂ and 95% humidity. Non-adherent cells were loaded directly into an operating sterilized elutriator system (Beckman Instruments, Palo Alto, CA) and cells were subsequently separated as follows. Elutriation was performed using a Beckman JE-6B rotor system equipped with a 40 ml chamber. Rotor speed was kept constant at 2040 rpm and cells were separated by increasing the flow rates. Cells ($1-9 \times 10^9$) were loaded at a flow rate of 45 ml/min. After all the cells had entered into the chamber, media flow rate was increased to 50 ml/min and a first fraction (700 ml) was collected. A second fraction (700 ml) was collected by increasing flow to 75 ml/min. After collecting the second fraction media flow and rotor were turned off and chamber contents were harvested aseptically in a biological hood. The 75 ml/min fraction was found to be enriched for early hematopoietic progenitors (CFU-mix and BFU-e) by in vitro bone marrow culture assays.

Cells from the 75 ml/min fraction were pelleted by centrifugation and resuspended in Dulbecco's phosphate buffered saline solution with calcium and magnesium containing 1% BSA (DPBS). Cell concentration was adjusted to 10^8 /ml. CD2+lymphocytes and mature myeloid cells were depleted using 2 murine anti-porcine monoclonal antibodies, MSA-4 and 74-22-15, both have been shown to bind mature porcine lymphocytes and myeloid cells, respectively. The antibodies were added to the cells at a concentration of 7 μ g each / 10^8 cells, then incubated at 4 C for 30 minutes. Cells were washed twice in cold DPBS and resuspended in 2ml of DPBS. Goat anti-mouse IgG Dynabeads (M450, Dynal, Oslo, Norway) were washed and resuspended in DPBS and added to the cell suspension. The ratio of beads to cells was 4×10^8 beads to 1×10^8 cells. The cell-bead mixture was incubated for 30

minutes at 4 C, after which the pellet was gently resuspended by pipetting. The cells attached to the beads were collected using the appropriate sized magnetic particle concentrator (MPC, Dynal) and the unattached beads are collected in the supernatant. The supernatant was transferred to a second test tube and replaced in the MPC to remove any residual cells attached to beads. Cells were counted, cytospin slide preparations were made and cells were put into CFU assays to evaluate the preparation.

RNA isolation

Total RNA was isolated from 1×10^7 enriched porcine bone marrow cells using the Micro-scale Total RNA Separator Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions. Poly A+ RNA was isolated from the above using the PolyAtract mRNA Isolation System (Promega, Madison, WI) according to the manufacturer's instructions.

Porcine genomic DNA preparation

Genomic DNA from miniswine peripheral blood mononuclear cells was prepared as previously described (Molecular Cloning: A Laboratory Manual, T. Maniatis et al. eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, NY).

Isolation of cDNA clones of the porcine CD34 gene

An overview of the process utilized to isolate the porcine CD34 cDNA gene is presented, followed by a detailed description. Briefly, clone pCRII/exon 8-#8 was generated from porcine genomic DNA using primer sequences derived from exon 8 of the human and mouse CD34 genes. Clone pCRII/exon 7-8 was generated from porcine bone marrow RNA using a human CD34 forward primer and nested porcine reverse primers derived from the sequence of pCRII/exon 8. Clone pCRII/9-13 was generated from bone marrow RNA using a human CD34 forward primer and nested porcine reverse primers derived from the

sequence of pCRII/exon 7-8. Clone 4-5-6 B was derived from porcine bone marrow RNA by 5' RACE using porcine CD34 reverse primers derived from the sequence of pCRII/9-13. Finally, porcine forward and reverse primers were used to derive clones 1AX2-3, 1BX2-4 and 1CX2-5 independently from bone marrow RNA. The details of these constructions follow.

General PCR Protocol

PCR reactions were run in 100 ul of Standard Reaction Mix (1X Buffer PCR II (Perkin-Elmer, Norwalk, CT), 100 uM dNTP, 3 mM MgCl₂, 25 u/ml AmpliTaq polymerase (Perkin-Elmer)) and both forward and reverse primers at 200 nM. The hot start method, using Ampliwax Beads (Perkin-Elmer) according to the manufacturer's instructions, was utilized. Reactions were run in a System 9600 Thermocycler (Perkin-Elmer).

pCRII/exon 8-#8

Forward primer mCD34-4 (SEQ ID NO:1; 5'-GGTGAAGACCCTTATTACACGG) was derived from the sequence of exon 8 of the murine CD34 gene (Brown J. et al., 1991, International Immunology 3:175) and reverse primer hCD34-2R (SEQ ID NO: 2, 5'-CGTGTGTGCTTGCTGAATGG) was derived from the sequence of exon 8 of the human CD34 gene (Simmons D.L. et al., 1992, J. of Immunology 148:267). 100 ng of porcine genomic DNA was added. The following amplification profile was used:

- 1) 94 C for 2 minutes
- 2) 39 cycles of:
 - a) 94 C for 45 sec
 - b) T anneal for 60 sec
 - c) 72 C for 2 minutes

where T anneal began at 55 C and decreased 1 C after every 3 cycles

- 3) 10 cycles of:

- a) 94 C for 45 sec
 - b) 42 C for 60 sec
 - c) 72 C for 2 minutes
- 4) Soak at 4 C.

Reaction products were cloned into the vector pCR II (In Vitrogen, San Diego, CA) according to the manufacturer's instructions to create clone pCRII/exon 8-#8. The insert of the clone was sequenced using the Sequenase Sequencing Kit (USB, Cleveland, OH) according to the manufacturer's instructions.

pCRII/exon 7-8

cDNA was prepared from enriched porcine bone marrow cell poly A+ RNA using the 1st STRAND cDNA Synthesis Kit (Clontech) according to the manufacturer's instructions. Forward primer hCD34-4 (SEQ ID NO:3; 5'-GCAAGCCACCAGAGCTATT) and reverse primer hCD34-2R (SEQ ID NO: 4; 5'-CGTGTTGTCTTGCTGAATGG), were both derived from the human CD34 sequence. cDNA derived from 25 ng of poly A+ RNA was added and the following profile used:

- 1) 94 C for 2 minutes
- 2) 36 cycles of:
 - a) 96 C for 2 sec
 - b) T anneal for 45 sec
 - c) 72 C for 1 minute

where T anneal begins at 60 C and decreased 1 C after every 3 cycles

- 3) Soak at 4 C

A secondary reaction was run with 5 ul of the above primary as template. Forward primer hCD34-4 and reverse primer pCD34-4R (SEQ ID NO:5; 5'-AGTCACACTGGCTTTTCCCTGA), were derived from the sequence of clone pCRII/exon 8-#8. The reaction profile used was:

- 1) 94 C for 2 minutes
- 2) 35 cycles of:
 - a) 96 C for 2 sec
 - b) 55 C for 45 sec
 - c) 72 C for 1 minute
- 3) Soak at 4 C

Cloning and sequencing of the secondary product was performed as above.

pCRII/9-13

Porcine bone marrow cDNA, prepared above, was used as template for a primary PCR reaction. Forward primer hCD34-1.1 (SEQ NO: 6; 5'-AGTACCCTTGGAAGTACCAGC), was derived from the sequence of human CD34, and reverse primer pCD34-5R (SEQ ID NO:7; 5'-AAGACAGCCAGCAGGATCC), was derived from the sequence of clone pCRII/exon 7-8. The following amplification profile was used:

- 1) 94 C for 2 minutes
- 2) 35 cycles of:
 - a) 96 C for 2 sec
 - b) 55 C for 50 sec
 - c) 72 C for 1 minute
- 3) Soak at 4 C.

A secondary reaction was run with 5 ul of the above primary as template. Forward primer hCD34-1.1 and reverse primer pCD34-6R (SEQ ID NO: 8; 5'-GGTGACCAGTGCAATCAAGG), derived from the sequence of clone pCRII/exon 8-#8, were used. The reaction was run as above. Cloning and sequencing of the secondary product was performed as above.

4-5-6 B

cDNA was prepared from oligo dT primed poly A+ RNA of progenitor-enriched porcine bone marrow using the 5' RACE System (Gibco) according to the manufacturer's instructions.

Following RNase H treatment, the cDNA was phenol/chloroform extracted and purified over a Size-Sep 400 Column (Pharmacia, Piscataway, NJ). The purified cDNA was then tailed with dCTP using terminal transferase from the 5' RACE System. A primary PCR reaction, containing tailed cDNA from approximately 40 ng of RNA, was run with the BRL anchor primer from the 5' RACE kit as forward primer and pCD-888 (SEQ ID NO: 9; 5'-GTGCAATCAAGGTCTTTCGG), derived from the sequence of clone pCRII/exon 7-8, as reverse primer. The following amplification profile was used:

- 1) 94 C for 2 minutes
- 2) 10 cycles of:
 - a) 96 C for 2 sec
 - b) 56 C for 30 sec
 - c) 72 C for 1 minute
- 3) 72 C for 5 minutes
- 4) Soak at 4 C.

The primary amplification reaction was purified free of primers and short amplification products over a cDNA Column (Pharmacia) according to the manufacturer's instructions. A secondary reaction contained 5 ul of the primary reaction product as template. The forward primer was MUAP (SEQ ID NO: 10; 5'-CTAGGCCACGCGTCGACTAGTAC), which overlaps the 5' RACE System anchor primer, and the reverse primer was pCD-662 (SEQ ID NO: 11; 5'-CCCACACAGGATTTGCATC), derived from the sequence of pCRII/9-13. The following amplification profile was used:

- 1) 94 C for 2 minutes
- 2) 35 cycles of:
 - a) 96 C for 2 sec
 - b) 56C for 30 sec
 - c) 72 C for 1 minute
- 3) 72 C for 5 minutes
- 4) Soak at 4 C.

A tertiary amplification reaction contained 5 ul of the secondary reaction product.. The forward primer was MUAP and the reverse primer pCD-604 (SEQ ID NO: 12; 5'-GAGGTCTCATTTTCGCTCCAG), derived from the sequence of pCRII/9-16. A product of 700-800 bp was isolated from this reaction on a low melting temperature agarose gel and cloned into the vector pCRII as previously. The sequence of the 5' end of this clone, including the presumptive translational initiation codon and 5' untranslated sequence, was obtained as previously and by using the fmol Thermocycle Sequencing Kit (Promega) according to the manufacturer's instructions.

1AX2-3, 1BX2-4 and 1CX2-5

Three independent primary PCR reactions were run, using the same untailed cDNA prepared for isolation of clone 4-5-6 B. The forward primer was PIG5 (SEQ ID NO:13; 5'-ATCTATCTCTTCGGAAGCGG), derived from the presumptive 5' untranslated region of porcine CD34 in clone 4-5-6 B. The reverse primer was pCD-974 (SEQ ID NO: 16; 5'-TTCTCCTGTAGGGCTCCAAC), derived from exon 7 sequences contained in clone pCRII/exon 7-8. The following amplification profile was used:

- 1) 94 C for 2 minutes
- 2) 35 cycles of:
 - a) 96 C for 2 sec
 - b) 56 C for 30 sec
 - c) 72 C for 1 minute
- 3) 72 C for 5 minutes
- 4) Soak at 4 C.

Secondary amplification reactions, for each of the three independent primary reactions, were run using 5 ul of the primary reaction as template. The forward primer was 5X (SEQ ID NO: 14; 5'-ATAGTTTAGCGGCCGCATCTATCTCTTCGGAAGCGG), which overlaps primer PIG5 and adds a Not I site to the end. The

reverse primer was 3X (SEQ ID NO: 15; 5'-CCTACAGGAGAAAGGCTGGAGCTGGAACCCCTGAGCGGCCGCTAAACTAT). This primer, which overlaps sequences in pCD-974, contains porcine sequence through the end of exon 7 (derived from clone pCRII/exon 7-8) followed by human sequence found in the alternatively spliced form of human CD34 (Nakamura Y., et al., 1993, Experimental Hematology 21:236); it also adds a Not I site to the end. The following amplification profile was used:

- 1) 94 C for 2 minutes
- 2) 7 cycles of:
 - a) 96 C for 2 sec
 - b) 56 C for 30 sec
 - c) 72 C for 1 minute
- 3) 72 C for 5 minutes
- 4) Soak at 4 C.

The resulting products were cleaved with Not I and cloned into the Not I site of vector pRcCMV (InVitrogen) using standard methodology. One clone, in proper orientation relative to the CMV promoter of pRcCMV, from each independent amplification was chosen for sequencing. Sequence of at least one strand of each of the clones 1AX2-3, a1BX2-4 and 1CX2-5 was obtained by Sequenase and fmol sequencing reactions.

Results:

The sequence of the CD34 insert of clone 1AX2-3 is shown in Figure 1, and is flanked by Not I cloning sites in the clone (not shown).

Nucleotides 1-20 of figure 1 are derived from amplification primer 5X; this sequence was originally derived from CD34 RACE clones 4-5-6 B. Nucleotides 1033-1065 are derived from amplification primer 3X. Nucleotides 1033-1050 encode the end of presumptive exon 7 of the porcine gene and were originally determined from porcine CD34⁺ clone pCRII/exon 7-8. Nucleotides 1051-1065, and correspondingly amino acids 310-313, may not match authentic porcine sequence.

The presumptive N-terminus of processed porcine CD34 is indicated with amino acid number 1. This assignment is made purely by homology to the known terminus of human CD34.

In two independently derived clones, there is an A nucleotide at position 202 and a G nucleotide at position 405; these differences may have arisen as *in vitro* artefacts in the PCR reactions used in the CD34 cloning or may result from polymorphisms within the porcine CD34 gene.

Identification of the sequence represented in Figure 1 as porcine CD34 is made by comparison of the nucleic acid and amino acid sequences with those of human CD34. The porcine portions of the nucleic acid sequence in Figure 1 are about 67% identical to human CD34, while the amino acid sequences are about 59% identical.

Example 2

Generation of anti-CD34 antibodies using soluble CD34 secreted from *Pichia pastoris*

Materials and Methods:

Construction of *Pichia pastoris* CD34 expression vector: A DNA fragment of the porcine CD34 cDNA sequence containing the mature coding sequence of CD34 minus the transmembrane domain is generated using PCR methodology. Primer mdr1 (5'-TGT CTA CTC GAG AAA AGA GAG GCT GAA GCT GTG AAC AGC TCA ACT ATT) contains an XhoI site at the 5' end followed by codons encoding Glu-Lys-Arg-*Glu-Ala-Glu-Ala where * is the site of cleavage for the KEX2 protease. Following the Glu-Ala sequence are the codons for the amino terminal sequence of mature CD34. The antisense primer mdr2 (5'-ACTAGAATTCTTATCA CTT TCG GGA GTA GCT CTG) encodes the amino acids of CD34 immediately preceding the transmembrane domain, two in-frame translation termination codons and an EcoRI site. The PCR product generated from amplifying the CD34 insert from plasmid 1AX2-3 using primers mdr1 and mdr2 is digested with XhoI and EcoRI and purified from an agarose gel using the QIAEX Gel Extraction Kit (QIAGEN Inc. Chatsworth, CA). The purified fragment is ligated into vector pPIC9 (InVitrogen Inc.) that has been cleaved with XhoI and EcoRI, treated with alkaline phosphatase and purified by gel electrophoresis. One clone, pMDR6295, is confirmed to have the correct DNA sequence and is used to transform *Pichia pastoris*, according to the instructions detailed in the *Pichia Expression Kit Manual of Methods* (InVitrogen Corp., San Diego, CA). Transformants are screened for His⁺Mut⁺ phenotype due to integration into the HIS4 locus. *Pichia* genomic DNA is prepared by the Easy-DNA Kit protocol (InVitrogen Corp., San Diego, CA) and analyzed by PCR as described in the instruction manual. One clone, MDR6295, is verified to

contain the CD34 insert and used to generate soluble secreted CD34 protein.

Example 3

Generation of Monoclonal Antibodies Using Mouse

L-cells Expressing Porcine CD34

Mammalian expression vector for L-cell transformation: Clone 1AX2-3, generated as described in Example 1, contains the CMV promoter for expression of the CD34 insert as well as an SV40-neo cassette for stable selection based on G418 resistance, and is therefore suitable as a mammalian expression vector.

L-cell Transformation: CHO and mouse L-cells are transformed by electroporation using methods previously published for transformation of CHO cells (J. Barsoum, 1990, DNA and Cell Biology 9:293). Briefly, 5×10^6 trypsinized cells are resuspended in 200 μ l of 1XHeBS (20 mM HEPES pH 7.05, 137 mM NaCl, 5 mM KCl, .7 mM Na_2HPO_4 , 6 mM dextrose) containing 50 μ g of Pvu I linearized plasmid 1AX2-3 and 50 μ g sheared salmon testes DNA. Electroporation is performed using a GenePulser apparatus (BioRad Laboratories, Hercules, CA) set at 290V and 250 μ FD for CHO cells or at 240V and 250 μ FD for L-cells cultured for 48-72 h prior to addition of G418 at 400 μ g/ml to the culture medium. After the appearance of discrete colonies, the cells are trypsinized and replated to create single polyclonal lines for CHO and L-cell transformants.

Example 4

Generation and Screening of Monoclonal

Antibody Producing Lines

Immunization of Mice: Immunization of mice with the soluble fragment of porcine CD34 described in Example 2 is performed essentially as described (Antibodies: A Laboratory Manual, E.

Harlow and D. Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). . Briefly, 50 ug of protein is injected intraperitoneally into BABL/C mice in complete Freund's adjuvant. A boost is given 10-14 days after the primary immunization as above but in incomplete Freund's adjuvant. Fusions are performed 5 days following the boost.

For mouse L-cell immunizations, 10^6 transformed cell (Example 3), rinsed and resuspended in PBS, are injected intraperitoneally into C3H x Balb C F1 hybrid mice. A boost is again given after 1-14 days, with fusions following 5 days after the boost.

Hybridoma Production: Hybridoma production followed standard protocols (Current Protocols in Immunology, eds. J.E. Coligan et al., Wiley and Sons, New York, NY). Splenocytes from immunized animals are fused to SP2/0-Ag14 myeloma cells and HAT selected culture wells tested for anti-CD34 antibodies.

Screening of hybridomas: For hybridomas raised against soluble CD34 produced in Pichia, wells are first tested by ELISA for binding to the same protein. Positive wells are additionally tested by FACS analysis using a polyclonal CHO cell line expressing porcine CD34 (Example 3) or the untransformed parental line.

For hybridomas raised against transformed L-cells, culture wells are tested for antibody against transformed and untransformed CHO cells as above.

Positive clones are finally tested by FACS analysis for binding to a small proportion (1-4%) of porcine bone marrow cells.

Positive wells are frozen, subcloned and antibody produced in vivo as ascites fluid or in vitro.

Numerous modifications and variations of the present invention are possible in light of the above teachings; therefore, within the scope of the appended claims, the invention may be practiced otherwise than as particularly described.

What Is Claimed Is:

1. An antibody which recognizes porcine CD34⁺ cells.
2. A process for recovering porcine CD34⁺ cells from a material containing such cells, comprising:
contacting a material which contains porcine CD34⁺ cells with the antibody of Claim 1.
3. The antibody of Claim 1 wherein said antibody recognizes the polypeptide of Figure 1.
4. A polypeptide encoded by the DNA contained in one of ATCC Deposit No. 97,143, 97,144, 97,145.
5. An antibody against the polypeptide of Claim 4.
6. Isolated porcine cells which are recognized by the antibody of Claim 5.
7. The cells of Claim 6 wherein the cells are isolated from porcine bone marrow.
8. A process for producing mixed chimerism in a primate, comprising:
administering to a primate the cells of claim 6.

1/2

Sequence of the CD34 insert of clone 1AX2-3

ATCTATCTCT TCGGAAGCGG CGCGGGAAGG 30

ATG CTG ATC CGC AGG GGC GCG CGC GCG GGG CGC GGG	ATG CCG CGG GGC TGG ACC ACG CTC TGC TTG	96
Met leu ile arg arg gly ala arg ala gly arg gly	met pro arg gly trp thr thr leu cys leu	
-30	-15	-10
CTG AGT TTG CTG CCC TCT GGG TTC ACA GCT GTG AAC AGC TCA ACT ATT GCT TCC ACC TTG	156	
leu ser leu leu pro ser gly phe thr ala val asn ser ser thr ile ala ser thr leu		
-5	1	10
CCA GCT GCC GCT GGG TCA ACT CCC ACC GGG CCG GCT ACC GCA GGG GCA GCT ATC ACC GGG	216	
pro ala ala ala gly ser thr pro thr gly pro ala thr ala gly ala ala ile thr gly		
15	20	25
TCA ACT ATC TCA GAC ATA TCT TCA CCT GTT TCT ACA AAT ATA TCC AAC GAG GAA ACC ACA	276	
ser thr ile ser asp ile ser ser pro val ser thr asn ile ser asn glu glu thr thr		
35	40	45
TCA GAT GCT TTC GAA AGT GCC AGC CTC CAC ACT GTC TCT CAG GGC AGC AGT GGG ACC ACC	336	
ser asp ala phe glu ser ala ser leu his thr val ser gln gly ser ser gly thr thr		
55	60	65
GTA GCC ATC TCA GGC CCT ACA GTT AAT TTC ATG TCT ACC TCG GCG GTC ACC CTC GTC CCC	396	
val ala ile ser gly pro thr val asn phe met ser thr ser ala val thr leu val pro		
75	80	85
GAA ACC GTT AAC TCT TCT GTC CAG CCT CAG ACC TCT CTA GCC ACA GCG TCC TCC GCC ACC	456	
glu thr val asn ser ser val gln pro gln thr ser leu ala thr ala ser ser ala thr		
95	100	105
ATC AAC TTT ACA ACT TCA GAG GTG ACC CTG CAG CCC AGC ACG TTC CCA GGA AAT GTT TCA	516	
ile asn phe thr thr ser glu val thr leu gln pro ser thr phe pro gly asn val ser		
115	120	125
GAC CCC CTC TAC AAC AGT ACC AGC CCT GCG AGA TCC CCC ACC AGC CCC TAC ACA TCA TCT	576	
asp pro leu tyr asn ser thr ser pro ala arg ser pro thr ser pro tyr thr ser ser		
135	140	145
CCT CCT ACC CCA GGT AGC CAC AAG GGG GAA GTC AAA TGT GCC CAA ATC AAA GAG GTG AAA	636	
pro pro thr pro gly ser his lys gly glu val lys cys ala gln ile lys glu val lys		
155	160	165
TTG ACC CAA GGT ATC TGC CTG GAG CGA AAT GAG ACC TCC GCC TGC GAG AAG TTT AAG AAG	696	
leu thr gln gly ile cys leu glu arg asn glu thr ser gly cys glu lys phe lys lys		
175	180	185
GAC AAT GGA GAG AAG TTG ATG CAA ATC CTG TGT GGG CAG GAG CAG GCT GAG CCC GGG CCA	756	
asp asn gly glu lys leu met gln ile leu cys gly gln glu gln ala glu ala gly pro		
195	200	205
GGG GTG TGC TCC TTG CTC CIT GCC CAA TCT GAG GTG AAA CCT CAC TGC CTG CTG CTG GTC	816	
gly val cys ser leu leu leu ala gln ser glu val lys pro his cys leu leu leu val		
215	220	225
TTG GCC AAC GGA ACA GAA CTT AGC AGC AAG TTC CTG CTT CTG GAA AAG CAC CAG TCT GAA	876	
leu ala asn gly thr glu leu ser ser lys phe leu leu leu glu lys his gln ser glu		
235	240	245
		250

FIGURE 1

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CTG AGA GAG ATG AGC ATC CAA AAC TTC TCG AAA CAA GAT GTT ACG AGC CAC CAG AGC TAC 936
 leu arg glu met ser ile gln asn phe ser lys gln asp val arg ser his gln ser tyr
 255 260 265 270

TCC CGA AAG ACC TTG ATT GCA CTG GTC ACC TCG GGG ATC CTG CTG GCT GTC TTG CCC ATC 996
 ser arg lys thr leu ile ala leu val thr ser gly ile leu leu ala val leu gly ile
 275 280 285 290

ACT GGC TAC TTG CTG ATG AAC CGT CGC AGT TGG AGC CCT ACA GGA GAA AGG CTG GAG CTG 1056
 thr gly tyr leu leu met asn arg arg ser trp ser pro thr gly glu arg leu glu leu
 295 300 305 310

GAA CCC TGA 1065
 glu pro TER

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08340

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : Please See Extra Sheet.

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG, BIOSIS, CA, EMBASE, MEDLINE, WPI

search terms: porcine, pig, swine, cd34, hemopoie?, stem, progenitor, xenograft

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A1, 93/09815 (THE GENERAL HOSPITAL CORPORATION) 27 May 1993, see entire document.	1-8
Y	Blood Cells, Volume 20, issued 1994, Emery et al., "Bone Marrow Culture And Transduction Of Stem Cells In A Minature Swine Model", pages 498-503, see entire document.	1-8
Y	Exp. Hematol., Volume 21, issued 1993, Nakamura et al., "Two Alternative Forms Of cDNA Encoding CD34". pages 236-242, see entire document.	1-8

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z* document member of the same patent family
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P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 14 AUGUST 1996	Date of mailing of the international search report 27 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer <i>Phillip Gambel</i> PHILLIP GAMBEL Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/08340

A. CLASSIFICATION OF SUBJECT MATTER:

IPC (6):

A61K 39/395; C07K 14/435, 14/47, 14/705, 16/00, 16/18, 16/28; C12N 15/12, 15/85; G01N 33/53

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

424/93.7, 93.7, 529, 534; 435/2, 7.1, 69.1, 172.3, 240.2, 240.21, 252.3, 320.1; 530/350, 387.1, 388.1, 388.22, 388.7, 389.1, 389.6; 536/23.1, 23.5

B. FIELDS SEARCHED

Minimum documentation searched

Classification System: U.S.

424/93.7, 93.7, 529, 534; 435/2, 7.1, 69.1, 172.3, 240.2, 240.21, 252.3, 320.1; 530/350, 387.1, 388.1, 388.22, 388.7, 389.1, 389.6; 536/23.1, 23.5

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